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# PER-8, a Novel Extended-Spectrum β-Lactamase PER Variant, from an *Acinetobacter baumannii* Clinical Isolate in Nepal

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**ABSTRACT** A novel PER-type extended-spectrum β-lactamase, PER-8, was identified in an *Acinetobacter baumannii* clinical isolate obtained in Nepal. The amino acid sequence of PER-8 has a substitution at position 39 (Gly to Glu) compared with that of PER-7. The $k_{cat}/K_m$ ratio of PER-8 for aztreonam was lower than that of PER-7, while the $k_{cat}/K_m$ ratio of PER-8 for imipenem was higher than that of PER-7. The genomic environment surrounding $bla_{PER-8}$ was *intI1* $bla_{PSE-1}$ *qacEΔI sulI* IS*CR1*-$bla_{PER-8}$ *gts sulI orfX* on a 100-kb plasmid.

**KEYWORDS** *Acinetobacter baumannii*, PER-type ESBLs, plasmid-mediated resistance

The class A extended-spectrum β-lactamases (ESBLs) confer resistance to expanded-spectrum cephalosporins and are inhibited *in vitro* by clavulanic acid and tazobactam (1). Resistance to broad-spectrum cephalosporins in *Acinetobacter baumannii* mostly results from overexpression of the natural AmpC-type enzyme or from acquisition of ESBLs. To date, the following 5 types of ESBL genes have been reported in *A. baumannii*: $bla_{PER}$ (2), $bla_{GES}$ (3), $bla_{VEB}$ (4), $bla_{TEM}$ (5), and $bla_{CTX-M}$ (6). The $bla_{PER-1}$ gene was first found in a *Pseudomonas aeruginosa* isolate (7). Since then, it has been reported worldwide in *Enterobacteriaceae* (8–11) and *A. baumannii* (2, 4). Until now, 7 types of PER variants have been reported in clinical isolates of *Enterobacteriaceae* (12–16) and *A. baumannii* (17) in various countries. The phylogenic tree based on amino acid sequences (Clustal W2) revealed two clusters in PER-type variants, one containing PER-1, PER-3, PER-4, PER-5, and PER-7 and the other containing PER-2 and PER-6.

Multidrug-resistant *A. baumannii* IOMTU442 and IOMTU448 were isolated from samples of wound swabs from hospitalized patients at a university hospital in 2013 in Nepal. The isolates were identified phenotypically, and species identification was confirmed by comparing sequences of 16S rRNA, *gyrB*, and $bla_{OXA-51-like}$ genes. *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and expression of $bla_{PER}$ genes, respectively.

MICs were determined using the broth microdilution method as recommended by CLSI (M100-S23). Whole genomes of IOMTU442 and IOMTU448 were extracted with DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego, CA). Multilocus sequence typing (MLST) was performed as described by the protocols of the Institut Pasteur MLST (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_abaumannii_pasteur_seqdef) databases.

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**TABLE 1** MICs of various β-lactams for *A. baumannii* strains IOMTU442 and IOMTU448 and *E. coli* DH5α transformed with PER-7- or PER-8-encoding plasmids

| | MIC (mg/liter) | | | | |
|---|---|---|---|---|---|
| **Antibiotic(s)[a]** | **IOMTU442** | **IOMTU448** | **pHSG398/PER-7** | **pHSG398/PER-8** | **pHSG398** |
| Amikacin | >1,024 | 8 | ND[b] | ND | ND |
| Ampicillin | >1,024 | >1,024 | 32 | 16 | 2 |
| Ampicillin-sulbactam | 64 | 64 | 2 | 2 | 2 |
| Arbekacin | >1,024 | 2 | ND | ND | ND |
| Aztreonam | >1,024 | >1,024 | 8 | 4 | ≤0.063 |
| Cefepime | 512 | 512 | 0.125 | 0.25 | ≤0.063 |
| Cefmetazole | 256 | >1,024 | 1 | 1 | 1 |
| Cefotaxime | 512 | >1,024 | 16 | 8 | ≤0.063 |
| Cefoxitin | 512 | >1,024 | 4 | 4 | 4 |
| Cefpirome | 256 | 256 | ≤0.063 | ≤0.063 | ≤0.063 |
| Ceftazidime | >1,024 | 512 | 32 | 64 | 0.5 |
| Cephradine | >1,024 | >1,024 | 128 | 128 | 16 |
| Ciprofloxacin | 32 | 32 | ND | ND | ND |
| Colistin | 0.25 | 0.5 | ND | ND | ND |
| Fosfomycin | 128 | 256 | ND | ND | ND |
| Gentamicin | >1,024 | >1,024 | ND | ND | ND |
| Imipenem | 2 | 8 | 0.125 | 0.125 | ≤0.063 |
| Kanamycin | >1,024 | >1,024 | ND | ND | ND |
| Levofloxacin | 32 | 8 | ND | ND | ND |
| Meropenem | 1 | 16 | ≤0.063 | ≤0.063 | ≤0.063 |
| Moxalactam | 128 | 128 | ≤0.063 | ≤0.063 | ≤0.063 |
| Penicillin G | >1,024 | >1,024 | 64 | 64 | 32 |
| Piperacillin | >1,024 | 512 | 4 | 4 | 2 |
| Piperacillin-tazobactam | 64 | 256 | 2 | 2 | 2 |
| Tigecycline | 0.25 | 0.5 | ND | ND | ND |

[a]The ratio of ampicillin to sulbactam was 2:1. The ratio of piperacillin to tazobactam was 4:1.
[b]ND, not determined.

The $bla_{PER-7}$ and $bla_{PER-8}$ were cloned into the corresponding sites of the pHSG398 vector plasmid (TaKaRa, Shiga, Japan) using the primer set EcoRI-PER-F (5′-GGGAATT CATGGAATTGCCCAATATTATG-3′) and PstI-PER-R (5′-AACTGCAGTCAGCGCAGCTTGTCG GCCAT-3′). *E. coli* DH5α was transformed with pHSG398-PERs, and the transformants were selected on chloramphenicol-containing plates (30 μg/ml).

The open reading frames of PER-7 and PER-8, without signal peptide regions, were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI, USA) using the primer set BamHI-TEV-PER-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGG AAACTGGCGAC-3′) and XhoI-PER-R (5′-ATCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′). The plasmids were used to transform *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA, USA). Recombinant PERs were purified, and initial hydrolysis rates were determined as previously described (18).

To determine the size of the plasmid harboring $bla_{PER-8}$, plasmid DNA in iOMTU442 was extracted and digested with S1 nuclease. Pulsed-field gel electrophoresis (PFGE) and Southern hybridization were performed. A probe for $bla_{PER}$ from IOMTU442 was amplified by PCR using the EcoRI-PER-F and PstI-PER-R primer set. A DNA plug of IOMTU448, digested with I-CeuI, was prepared, separated by pulsed-field gel electrophoresis, and subjected to Southern hybridization using 16S rRNA and $bla_{PER-7}$ probes. Signal was detected using digoxigenin (DIG) High Prime DNA labeling and detection starter kit II (Roche Applied Science, Indianapolis, IN, USA).

IOMTU442 had $bla_{OXA-70}$, $bla_{PSE-1}$, and a novel $bla_{PER}$ variant, $bla_{PER-8}$. IOMTU448 had $bla_{OXA-23}$, $bla_{OXA-371}$, $bla_{OXA-420}$ ($bla_{OXA-58-like}$), and $bla_{PER-7}$. The $bla_{OXA-371}$ gene in IOMTU 448 was grouped with the $bla_{OXA-69}$-type genes, whereas the $bla_{OXA-70}$ gene in IOMTU442 was not grouped with the $bla_{OXA-66}$-type genes, the $bla_{OXA-69}$-type genes, or the $bla_{OXA-71}$-type genes. Neither $bla_{OXA-70}$ nor $bla_{OXA-371}$ was flanked by IS*Aba1*. The MICs for *A. baumannii* IOMTU442 and IOMTU448 are shown in Table 1. IOMTU442 and IOMTU448 were found to belong to ST103 and ST623, respectively. *A. baumannii* isolates belonging to ST103 have been found in Egypt (19) and Portugal (20), and ST623

**TABLE 2** Kinetic parameters of the PER-7 and PER-8 enzymes in hydrolyzing β-lactams[a]

| β-Lactam | PER-7 | | | PER-8 | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM)[b] | $k_{cat}$ ($s^{-1}$)[b] | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | $K_m$ (μM)[b] | $k_{cat}$ ($s^{-1}$)[b] | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) |
| Ampicillin | 13 ± 4 | 52 ± 2 | 4.4 | 19 ± 3 | 54 ± 1 | 2.9 |
| Penicillin G | 13.7 ± 2.8 | 16.0 ± 0.2 | 1.2 | 13.7 ± 3.1 | 16.4 ± 0.3 | 1.2 |
| Piperacillin | 12.6 ± 3.6 | 0.60 ± 0.02 | 0.051 | 8.6 ± 1.8 | 0.65 ± 0.03 | 0.076 |
| Cefepime | 81 ± 7 | 10.2 ± 0.3 | 0.13 | 110 ± 16 | 12 ± 1 | 0.11 |
| Cefmetazole | NH[c] | NH | NH | NH | NH | NH |
| Cefotaxime | 138 ± 63 | 84 ± 20 | 0.65 | 114 ± 17 | 70 ± 4 | 0.62 |
| Cefoxitin | NH | NH | NH | NH | NH | NH |
| Ceftazidime | 258 ± 22 | 33 ± 2 | 0.13 | 212 ± 26 | 31 ± 2 | 0.15 |
| Cephradine | 54 ± 5 | 62 ± 2 | 1.2 | 48 ± 8 | 66 ± 2 | 1.4 |
| Moxalactam | NH | NH | NH | NH | NH | NH |
| Aztreonam | 17 ± 3 | 8.8 ± 0.2 | 0.54 | 14 ± 2 | 8.8 ± 0.2 | 0.64 |
| Imipenem | 172 ± 33 | 0.13 ± 0.01 | 0.00076 | 101 ± 10 | 0.16 ± 0.01 | 0.0016 |
| Meropenem | 28 ± 8 | 0.13 ± 0.01 | 0.0051 | 32 ± 3 | 0.17 ± 0.01 | 0.0053 |

[a]The proteins were initially modified by the use of a His tag, which was removed after purification.
[b]$K_m$ and $k_{cat}$ values represent the means ± standard deviations of results of 3 independent experiments.
[c]NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

belonged to CC1, which is known as international clone I, disseminated worldwide. The sequence of $bla_{PER-8}$ showed a nucleotide substitution compared with $bla_{PER-7}$. Similarly, analysis of their predicted amino acid sequences revealed that PER-8 had a substitution (Gly39Glu) compared with PER-7; therefore, PER-7 was used as a control for PER-8. The nucleotide sequences of $bla_{PER-8}$ and its flanking region have been deposited in GenBank under accession number AB985401.

Compared with *E. coli* DH5α harboring a pHSG398 control vector, DH5α harboring $bla_{PER-7}$ or $bla_{PER-8}$ showed significantly increased MICs of all penicillins and cephalosporins tested, except cefmetazole, cefpirome, cefoxitin, and piperacillin, as well as slightly increased MICs of imipenem (Table 1). DH5α harboring $bla_{PER-7}$ and $bla_{PER-8}$ had similar MICs of β-lactams (Table 1). Recombinant PER-7 and PER-8 hydrolyzed all β-lactams tested, except for cefmetazole, cefoxitin, and moxalactam (Table 2). PER-7 and PER-8 also hydrolyzed imipenem and meropenem, although their $k_{cat}/K_m$ ratios against these substrates were quite low. The kinetic profiles of PER-8 against the β-lactams tested, except for imipenem, were similar to those of PER-7. The $k_{cat}/K_m$ ratios of PER-8 were 2-fold higher for imipenem than those of PER-7 (Table 2).

PFGE analysis showed that $bla_{PER-7}$ in IOMTU448 was located on the chromosome, whereas $bla_{PER-8}$ in IOMTU442 was located on a 100-kb plasmid whose replicon type was classified into the GR12 type of *Acinetobacter* plasmids (21). The genomic environments surrounding $bla_{PER-7}$ in IOMTU448 and $bla_{PER-8}$ in IOMTU442 are shown in Fig. 1. The genomic environments surrounding $bla_{PER-7}$ (nucleotide [nt] 1162 to nt 8196; GenBank accession no. LC020101) showed 99.4% nucleotide sequence identity with the region from nt 2172 to nt 9204 of the plasmid of *P. aeruginosa* RJ248 producing PER-1 in China (GenBank accession no. KU133340). The genomic environment surrounding $bla_{PER-8}$ (from nt 1994 to nt 8195; GenBank accession no. AB985401) showed more than 99.9% nucleotide sequence identity with the region from nt 3083 to nt 9284 of the plasmid from *A. baumannii* A068 producing PER-7 in Sweden (GenBank accession no. KT317086). $bla_{PER-7}$ and $bla_{PER-8}$ were both located downstream of IS*CR1* and had identical genetic structures for the sequence between *qacEDI* and *orfX* (*ofrX* is a gene encoding a putative ABC transporter ATP-binding protein) in their respective plasmids (Fig. 1). In our previous study, we reported PER-7-producing *A. baumannii* IOMTU433 in 2015 in Nepal (22). The structures upstream of the 3′ coding sequence (CS) in IOMTU442 and IOMTU448 completely differed from the corresponding regions of a plasmid (pIOMTU433) in *A. baumannii* IOMTU433 (22) (Fig. 1).

*A. baumannii* harboring $bla_{PER}$ genes, including $bla_{PER-7}$ and $bla_{PER-8}$, mediated by plasmids or chromosomes may be spreading in medical settings in Nepal, because our previous study showed that 49.2% of *A. baumannii* clinical isolates in Nepal harbored $bla_{PER}$ genes, including $bla_{PER-7}$ and $bla_{PER-8}$ (22). The $bla_{PER-7}$ gene was first identified

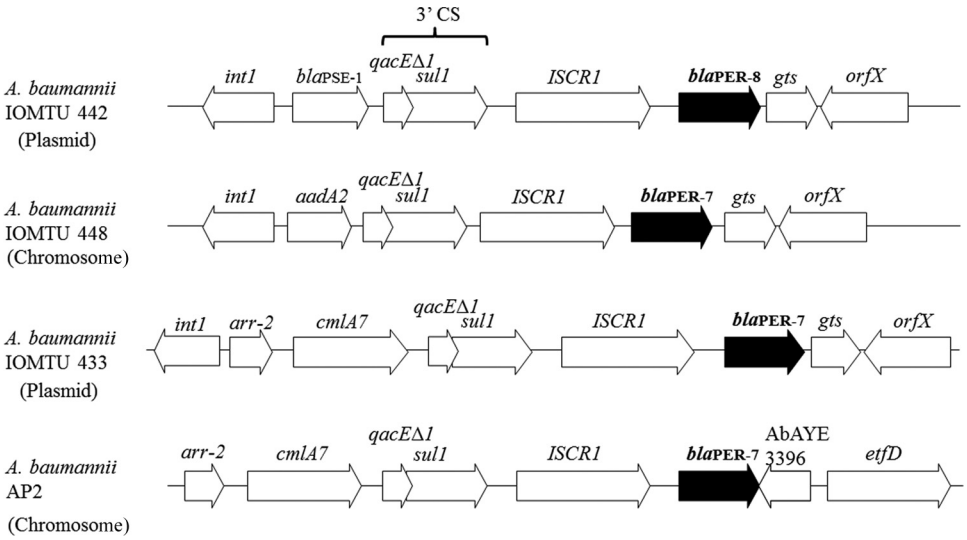


**FIG 1** Genetic environments surrounding $bla_{PER}$ genes in *A. baumannii* IOMTU442 (GenBank accession no. AB985401), IOMTU448 (GenBank accession no. LC020101), IOMTU433 (GenBank accession no. AP014650) (22), and AP2 (GenBank accession no. HQ713678) (17). The $bla_{PER-7}$ gene in *A. baumannii* AP2 was located on the chromosome, whereas the $bla_{PER-7}$ gene in *A. baumannii* IOMTU433 and the $bla_{PER-8}$ gene in *A. baumannii* IOMTU442 were located on plasmids.

in *A. baumannii* AP2 (GenBank accession no. HQ713678) in France, and the gene was located on the chromosome (17). As shown in Fig. 1, the genetic structures surrounding $bla_{PER}$ genes in IOMTU442 and IOMTU448 differ from that in AP2 because both IOMTU442 and IOMTU448 harbor *intI1* in the region upstream of $bla_{PER-7}$ and $bla_{PER-8}$, respectively, but AP2 does not. The upstream region of $bla_{PER-7}$ in *A. baumannii* AP2, *arr-2 cmlA7 qacED1 sull* IS*CR1*, had a structure identical to that in pIOMTU433 in *A. baumannii* IOMTU433 discovered in Nepal. The data from our present study suggest that PER-producing *A. baumannii* in Nepal probably has at least two types of genetic structures surrounding $bla_{PER}$ genes.

The insertion element IS*CR1* in the upstream region of $bla_{PER}$ genes appears to be involved in the acquisition of $bla_{PER}$ genes in *A. baumannii* in Nepal. The structure that includes 3′ CS-IS*CR1* is commonly associated with the recent emergence of drug-resistant pathogens, including *E. coli*, *Klebsiella pneumoniae*, *A. baumannii*, and *P. aeruginosa*, which are linked to the drug resistance genes encoding not only metallo-$\beta$-lactamases but also 16S rRNA methylases (23). The IS*CR1* may be associated with the genetic diversity of a $\beta$-lactamase-resistant factor in *A. baumannii* (24).

The $bla_{OXA-70}$ gene in IOMTU442 was first identified in *A. baumannii* clinical isolates in Hong Kong (25), whereas the $bla_{OXA-371}$ gene in IOMTU448 was first identified in *A. baumannii* clinical isolates in 2014 in Nepal (22). To date, $bla_{OXA-70}$ harboring *A. baumannii* was reported in 2014 in Canada (26). The $bla_{OXA-70}$ gene had 11, 17, and 17 nucleotide substitutions compared with $bla_{OXA-71}$, $bla_{OXA-66}$, and $bla_{OXA-69}$, respectively. The $bla_{OXA-371}$ gene had only one nucleotide substitution compared with $bla_{OXA-69}$.

In conclusion, this is the first report of *A. baumannii* isolates producing PER-7 and PER-8 in Nepal. The results of the present study indicate that plasmid- or chromosome-mediated PER-producing *A. baumannii* strains will spread in medical settings in Nepal.

**Accession number(s).** The nucleotide sequences for $bla_{PER-8}$ and its flanking region in *A. baumannii* IOMTU442 and for $bla_{PER-7}$ and its flanking region in *A. baumannii* IOMTU448 have been deposited in the GenBank database under accession numbers AB985401 and LC020101, respectively.

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